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(54) Title: INHIBITION OF HUMAN ROTAVIRUS INFECTION			
(57) Abstract <p>An enteral nutritional product containing either bovine κ-casein or human κ-casein at a concentration greater than that found in human or bovine milk and sufficient to inhibit infection of mammalian cells by human rotavirus.</p>			

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INHIBITION OF HUMAN ROTAVIRUS INFECTION

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a secretory protein, kappa-casein (κ -casein), which inhibits the attachment of human rotavirus (HRV) to mammalian cells.

Description of the Prior Art

Rotaviruses are the most important viral agents causing gastroenteritis in children living in both developing and developed countries (Volken et al, "Human Milk Mucin Inhibits Rotavirus Replication and Prevents Experimental Gastroenteritis", Journal of Clinical Investigation 90, 1984-1991, 1992). Rotaviruses also cause diarrhea in nursing homes and day care centers, among travelers, in adults who have contact with children, and in immunocompromised patients.

Breast feeding provides some protection against enteric infections by pathogens when breast-fed infants are compared with bottle-fed babies. Studies of children living in developing and developed countries have shown that breast-fed infants have fewer episodes of gastroenteritis than bottle-fed infants. Breast feeding can also lessen the severity of diarrhea and vomiting associated with enteric diseases. However, breast-feeding does not provide total protection against infection and rotavirus infection has been observed in breast-fed infants.

No single factor has been found to be entirely responsible for the protective effect of breast feeding. It is believed that antibodies play a role, however, the level of anti-rotavirus antibody in human milk does not correlate with the degree of protection afforded by the milk. This suggests that non-immunoglobulin factors contribute to the protective effect of breast milk, and investigations have been undertaken to identify these factors.

Volken et al. report a nonimmunoglobulin natural substance, milk mucin, that inhibits the replication of rotavirus. Mucin is a sialic acid-containing glycoprotein. In this report, Volken et

al. demonstrate that mucin and mucinous components found in human milk show anti-rotaviral activity. Using purified milk mucin components in a solid-phase binding assay, Yolken et al. first showed mucin binding to African Green Monkey Kidney (MA-104) cells infected with human and simian strains of rotavirus. Using tissue culture techniques they next showed that a fraction of human milk, subsequently identified as human milk mucin complex, inhibited rotavirus replication. This was further demonstrated by showing that this fraction bound specifically to cells infected with rotavirus. When the active fraction was desialylated by chemical hydrolysis, there was a substantial decrease in binding to the rotavirus infected cells. This indicates that in the antiviral system reported here sialic acid is necessary for anti-rotaviral activity. Yolken et al. did not find evidence of rotavirus inhibitory activity in the lipid fractions of milk.

Yolken et al. also demonstrated the efficacy of mucin-containing milk components in preventing experimental rotavirus gastroenteritis in suckling mice.

WO 94/09651 (Newburg et al., "Anti-Diarrheic Product and Method of Treating Rotavirus-Associated Infection") discloses a product containing an anti-diarrheal agent, such as the milk mucin complex and its components, which can bind rotavirus, and a method of inhibiting rotavirus infection in mammalian cells by exposing them to these agents.

U.S. Patent No. 5,147,853 (Dosako et al., "Infection Protectant") discloses a method for preventing adhesion of *E. coli* to human epithelial cells by treating them with κ -casein, a sialic acid-conjugated protein derived from cow's milk.

The prevalence of rotavirus infection in the groups at risk, especially infants and children, and the seriousness of its effects attests to the need for an effective treatment. It is of particular importance to develop methods for prevention and treatment of rotavirus infection that have no adverse side effects.

BRIEF DESCRIPTION OF THE INVENTION

In the present invention it is disclosed that a secretory protein, κ -casein, which can be purified from human or bovine milk, or manufactured in recombinant form using cDNA, inhibits the attachment of human rotavirus to mammalian cells. In an assay, 60-70% inhibition of human rotavirus binding was obtained when the cells were treated with either human or bovine κ -casein. κ -casein, which is a mammary product believed to be without adverse side effects, can be used for the prevention and treatment of rotavirus infection.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the method of preparation of radioactive iodine labeled human rotavirus.

Fig. 2 is a graph showing the extent of inhibition of binding by human rotavirus to MA-104 cells by human and bovine κ -caseins,

Fig. 3 shows the dose-dependent inhibition of human rotavirus to MA-104 cells by human κ -casein.

Fig. 4 shows that human κ -casein retained its ability to inhibit binding by human rotavirus to MA-104 cells after being treated with either neuraminidase, fucosidase or both.

Fig. 5 shows that bovine κ -casein retained its ability to inhibit binding by human rotavirus to MA-104 cells after being treated with neuraminidase.

Fig. 6 shows the effect of digestion by Pronase® on the ability of bovine κ -casein to inhibit binding by human rotavirus to MA-104 cells.

Fig. 7 shows the dose-dependent neutralization of human rotavirus as measured by the ability of bovine κ -casein to inhibit infection of MA-104 cells.

Fig. 8 shows the DNA sequence and the amino acid sequence of human κ -casein.

Fig. 9 shows the amino acid sequence which has the biological activity of human κ -casein.

DETAILED DESCRIPTION OF THE INVENTION

This invention results from a research effort directed toward improving methods for preventing and treating enteric infections caused by rotavirus in mammals, especially in humans. The invention demonstrates the effectiveness of κ -casein in inhibiting the attachment of human rotavirus (HRV) to simian cells, and thereby preventing the virus from entering the cells and causing infection. The agent of this invention, κ -casein, is particularly useful in the treatment of infants and children as it is a normal constituent of human milk and is present in the human diet in bovine milk. It is, thus, unlikely to cause toxic or allergic reactions in treated subjects.

Bovine κ -casein is present in bovine milk at a concentration of about 3.3 grams/liter. It can be added to food preparations and made available to both adults and children at risk for rotavirus infection with little, if any, risk of immunological reaction.

What is disclosed in this application is a method of preventing or retarding the onset of or treating an infection of a mammalian cell caused by human rotavirus comprising contacting the cell with an effective amount of an agent selected from the group consisting of native or recombinant unhydrolyzed human kappa-casein and native or recombinant unhydrolyzed bovine kappa-casein. Also disclosed is a method of preventing or retarding the onset of or treating human rotavirus infection of a mammal's cells comprising administering to the mammal an enteral nutritional composition comprising an anti-rotaviral infection effective amount of an agent selected from the group consisting of native or recombinant unhydrolyzed human kappa-casein and native or recombinant unhydrolyzed bovine kappa-casein at a concentration exceeding that found in human or bovine milk.

Specific examples will now be provided to describe the methods of preparation of κ -casein and the two assays employed to measure inhibition of HRV-binding ability by human and bovine κ -casein. The examples are provided for purposes of illustration

only and are not intended to be limiting.

Example 1: Preparation of Human Milk Kappa-casein

Approximately 0.5 l of frozen human milk was thawed, centrifuged at 15,000 x g for 1 hour at 4°C and the fat pad, which is the top layer, was removed. The supernatant and pellet were homogenized and the pH of the resulting material was adjusted to 4.3 by the addition of hydrochloric acid. Calcium chloride was added to a final concentration of 60 mM. The mixture was stirred at room temperature for 1 hour and the precipitated caseins were then recovered by centrifugation at 18,000 x g for 90 minutes at 4°C. Casein pellets were then dissolved in approximately 90 ml of 6M urea and 20 mM ethanolamine at a pH of 9.5 and extracted three times with 4 volumes of hexane to reduce the fat content further. The aqueous phase was dialyzed three times against 4 l of water using a 12,000 molecular weight cut-off membrane and lyophilized. This crude casein (yield was approximately 2-3 g/l) was dissolved in 6M urea, 20 mM n-2-hydroxyethylpiperazine-n'-2-ethane sulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, Mo) with 0.5% (V/V) 2-mercaptoethanol (2-ME) at pH 7.5 and stirred at 4°C for 1 hour. This solution was chromatographed in a 2.6 x 13 cm Macroprep-S® column (Bio-Rad, Hercules, CA) and equilibrated in the same buffer except that 0.1% (V/V) 2-ME was used. The washing buffer was 6M urea, 20 mM HEPES and 0.1% (V/V) 2-ME at pH 7.5. The eluant was monitored at a wavelength of 280 nm. Elution of the bound material was accomplished by application of a linear gradient from 0 to 0.3M sodium chloride in 100 minutes at a flow rate of 2 ml/minute. Fractions of 7 ml were collected and κ-casein was detected either by comparison with human κ-casein obtained from SYMBICOM AB, Sweden used as a standard in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or by immunoblot analysis. The fractions containing κ-casein were then pooled and dialyzed three times in 4 l of water as described above followed by lyophilization. This material was dissolved in

6M urea, 20 mM HEPES and 0.5% (V/V) 2-ME at pH 7.0 at a concentration of approximately 10 mg/ml at 4°C and loaded on the same column. The column was washed with 6M urea, 20 mM HEPES and 0.1% (V/V) 2-ME at pH 7.0 and then eluted with a linear gradient of 0 to 0.6M sodium chloride in 140 minutes at a flow rate of 2 ml/minute. The fractions containing the major peak at 280 MM were pooled, dialyzed as before and then lyophilized. The material was estimated to be of greater than 90% purity.

Example 2: Source of bovine Kappa-casein

Bovine κ -casein was purchased from Sigma (catalog #C0406) and had a purity of greater than 80%.

Example 3: Procedure for human rotavirus cell-binding inhibition assay

MA-104, African Green Monkey Kidney, cells (American Type Culture Collection, MD) were grown on 24-well tissue culture plates to 100% confluency. The use of Ma-104 cells is described in Kitamoto et al., "Comparative Growth of Different Rotavirus Strains in Differentiated Cells", Virology 184:729-737 (1991). Experiments reported by Kitamoto et al. show that human rotaviruses, including the Wa strain, grow and produce antigen in MA-104 cells. The cells were washed twice with Hanks' medium (Sigma) and fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 minutes. This was followed by three washes with tris-buffered saline (TBS). Solutions of potential inhibitors in TBS containing 5 mg/ml of bovine serum albumin (BSA) were added to the wells prior to the addition of ^{125}I -labeled human rotavirus (HRV) suspensions. Radiolabeled virus was prepared as described in Fig.1. The plates were incubated for 90 minutes at 37°C and washed three times with TBS. The cells were then lysed by adding 0.2% sodium hydroxide and 2% SDS solution. Counts per minute (cpm) in the lysates were determined in a scintillation counter and used as indicators of cell-bound HRV.

Example 4: Determination of Inhibition of Binding of Human Rotavirus Strain Wa to MA-104 Cells by Human and Bovine Kappa-Caseins

In an experiment shown in Fig. 2, five agents were tested for inhibitory potential at a concentration of 1 mg/ml. Human κ -casein inhibited binding by HRV strain Wa to MA-104 cells by more than 70% and bovine κ -casein (Sigma) inhibited binding by more than 60% when compared with control cells which did not receive any potential inhibitory agent after being exposed to HRV. Bovine beta-casein (Sigma) and human beta-casein (Symbicom AB, Sweden) exhibited much less ability to inhibit binding by HRV Wa. Bovine beta-casein showed 45% inhibition and human beta-casein showed 25% inhibition. Bovine submaxillary mucin (Sigma) was tested in the same experiment and showed 50% inhibition, however, further experimentation showed that this inhibition was not dose-dependent and, therefore, not specific. This is illustrated in Fig. 3. By contrast, human κ -casein was shown to be dose-dependent in the same experiment.

Example 5: Inhibition of Human Rotavirus Infection by Human or Bovine Kappa-Casein - Effect of Sialic Acid

In another experiment using the procedure described in Example 3, human κ -casein was treated with neuraminidase to remove sialic acid from the glycan chains of the protein molecules. As illustrated in Fig. 4 the desialylated human κ -casein showed similar activity in the rotavirus inhibition assay to the intact human κ -casein. This indicates that in this experiment the sialic acid residues are not required for the inhibitory effect of human κ -casein. Intact and desialylated human κ -casein were then treated with fucosidase. Both treated agents retained their inhibitory activity, which is indicated in Fig. 4. This shows that under the conditions of this assay fucose residues on human κ -casein are not required for inhibitory activity against HRV.

When bovine κ -casein was treated with neuraminidase to

remove sialic acids, the resulting desialylated bovine κ -casein was highly active in this assay, as shown in Fig. 5. It is concluded that sialic acid residues on bovine κ -casein are also not required for activity against HRV.

Example 6: Inhibition of Human Rotavirus Infection by Bovine Kappa-Casein - Effect of Hydrolysis

Bovine κ -casein was digested in 100-fold excess with a protease, Pronase® (Calbiochem, La Jolla, CA), for 16 hours at 37°C in 0.1M tris buffer at pH 8.0. Fig. 6 shows that the dose-dependent activity against HRV of the hydrolyzed protein was significant in this assay. The doses in this experiment were determined by the molar amounts of hexose present in the glycan chains of bovine κ -casein. Although 7-8 fold more of the hydrolyzed bovine κ -casein than native bovine κ -casein was required to achieve the same degree of inhibition, inhibitory activity against HRV was retained in the hydrolyzed protein under the conditions of this assay.

Example 7: Inhibition of Human Rotavirus Infection - Second Assay Method - MA-104 Cells and Wa Strain of Human Rotavirus

Another set of experiments was performed to measure the ability of bovine κ -casein to inhibit infection of cells by HRV. The assay procedure employed is as follows.

MA-104, African Green Monkey Kidney Cells whose use in rotavirus assays is described in Kitamoto et al, "Comparative Growth of Different Rotavirus Strains in Differentiated Cells", Virology 184:729-737 (1991), were obtained from BioWhittaker, Inc. (Walkersville, MD). Experiments reported by Kitamoto et al. show that human rotaviruses, including the Wa strain, grow and produce antigen in MA-104 cells. The cells were cultured in Basal Medium Eagle (BME) (BioWhittaker) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, UT) and 2 mM L-glutamine (BioWhittaker). Cells were

routinely subcultured in 75 cm² flasks using trypsin-EDTA (0.25% trypsin, 1 Mm EDTA) (BioWhittaker) to detach cells.

Assay plates were treated with 1 µg/well of fibronectin (Sigma) to aid in the adhesion of cells to the culture substrate and to ensure their subsequent attachment during the wash steps of the virus neutralization assay. Fibronectin, solubilized in distilled water, was dispensed into each well and incubated for 1-3 hours at room temperature to allow binding to the plastic wells. Prior to dispensing the cells into the wells of the assay plate, the residual fibronectin was aspirated. Cells seeded into 96-well plates at a density of 10,000 cells per well with growth medium were maintained at 37°C in a 5% CO₂ incubator for 3-4 days providing a confluent monolayer for virus neutralization studies.

Serotype 1 Wa strain of human rotavirus was obtained from Dr. Linda J. Saif (Ohio Agricultural Research and Development Center and was originally acquired from Dr. R. Wyatt (National Institutes of Allergy and Infectious Diseases). The virus stock used in these studies was a passage 5 viral harvest. Before use in these assays, HRV was activated with 10 IU/ml trypsin per 1 ml HRV and incubated for 30 minutes at 36°C. The virus was diluted with BME medium supplemented with 2 mM glutamine, 50 µg/ml gentamicin sulfate (BioWhittaker) and 1% penicillin-streptomycin solution (Sigma) to achieve a 1:250 dilution, which is twice the final desired virus concentration.

Example 8: Inhibition of Human Rotavirus Infection - Second Assay
Method - Virus Neutralization

Equal volumes of test agent and trypsin-activated HRV were mixed and preincubated for 1 hour at room temperature to permit the test agent to bind to the virus. During the HRV+test agent preincubation period, plates containing MA-104 cells were washed 3 times with 15 µl diluent medium (150 µl/well/wash) after which another 50 µl of diluent medium was added to each well. MA-104 plates were then incubated at 37°C in 5% CO₂ until the preincubation period for the HRV+test agent was completed. A

control sample or 100 μ l of the HRV+test agent sample was pipetted into appropriate wells and mixed with 50 μ l of diluent medium. The plates were incubated for an additional 12-14 hours at 37°C to allow the virus to propagate within the cells. Longer incubation time would result in infection of MA-104 cells by progeny virus, which is not desirable. Three types of control wells were included in each assay; (1) wells containing MA-104 cells, HRV, but no test agent, (2) wells containing MA-104 cells, HRV, and HRV antibodies, which are known HRV inhibitors and (3) wells containing MA-104 cells, but without virus. Each concentration of test agent or antibody was tested in triplicate.

After the incubation period, the assay plates were washed twice with Dulbecco's phosphate buffered saline (PBS) (BioWhittaker) 150 μ l of cold 70% ethanol was added to each well and 95 μ l was removed to prevent air from drying the cell monolayer. Cold absolute ethanol (190 μ l) was added to the 70% alcohol layer and the plates were rocked and refrigerated overnight. The alcohol was then removed and 150 μ l of PBS containing 0.05% chick egg albumin (PBS-CEA) (Sigma) was added to each plate to block nonspecific binding sites.

To stain the plates, the PBS-CEA was removed and 200 μ l of anti-rotavirus IgG was added to each well at a dilution of 1:2500. The plates were incubated for 30 minutes at room temperature. The plates were washed three times with PBS-CEA and 200 μ l of peroxidase conjugated to anti-bovine IgG (Cappel Organon Teknika, Durham, NC) was added to each well at a dilution of 1:2000. The plates were covered and incubated for 30 minutes at room temperature. They were then washed three times with PBS-CEA and 100 μ l of diaminobenzidine (Sigma) substrate was added to each well. The plates were incubated at room temperature for 20 minutes, washed twice with PBS-CEA and then washed with distilled water.

Example 9: Inhibition of Human Rotavirus Infection by Bovine
Kappa-Casein - Second Assay Method - Counting Infected
Cells

Plates which had been prepared according to the method of examples 7 and 8 were rehydrated by the addition of 150-200 μ l of distilled water per well prior to microscopic examination. The plates were examined with a light microscope at 100 \times magnification. Cells which were darkly stained and contained characteristic brown and granular cytoplasmic regions were counted as virus-infected cells. A representative infected cell count was obtained for each well by counting all the stained cells within a uniform area which represented approximately 20% of the surface area of each well. Control wells, to which no virus had been added, were examined to ascertain that they were free of infected cells. Results were reported as percent inhibition obtained by comparing the average number of infected cells in the population which had been treated with test agent+HRV with the average number of infected cells in the population which had been exposed to virus alone. Results are shown in Fig. 7. Bovine κ -casein, the test agent, neutralized human rotavirus in a dose-dependant manner. Increased concentrations of bovine κ -casein resulted in correspondingly reduced levels of infection up to a concentration of 1.6 mg/ml where infection was inhibited by almost 90% and only 10% of cells were infected.

Example 10: Inhibition of Human Rotavirus Infection by Bovine Kappa-Casein - Second Assay Method-Effect of Hydrolysis

Bovine κ -casein was digested with Pronase[®] (Calbiochem), a protease, for either 4 hours or 20 hours and at the concentrations indicated in Tables 1 and 2, which show experiments run on different days. The plates were incubated at 37°C according to the method of Example 8, and stained and counted according to the method of Example 9 to determine percent inhibition. The results of experiments performed on different days shown in Tables 1 and 2 indicate that hydrolysis of bovine

κ -casein with the protease eliminated inhibition of infection of MA-104 cells by HRV. Undigested bovine κ -casein showed between 86% and 93% inhibition of viral attachment to Wa cells. Hydrolyzed bovine κ -casein showed either no inhibition or very slight inhibition in this assay. Pronase® alone, without the addition of bovine κ -casein to the plates, resulted in variable amounts of inhibition which were not dose-related.

In this assay, bovine κ -casein with Pronase® or Pronase® alone were boiled after the period of digestion to denature the protein and abolish its activity. Therefore, all inhibition of HRV which has been observed can be attributed to the action of the bovine κ -casein and not to the Pronase®. In the assay described in Example 6, Pronase® was not boiled sufficiently boiled after the initial digestion period. The results of that assay showed that hydrolysis did not abolish inhibition. It is likely that in that case the protease had not been completely inactivated and the inhibition observed may be attributed to residual Pronase® rather than to hydrolyzed bovine κ -casein.

Table 1

INHIBITION OF HUMAN ROTAVIRUS INFECTION BY BOVINE KAPPA-CASEIN:
EFFECT OF HYDROLYSIS¹

TREATMENT	CONCENTRATION mg/ml	PERCENT INHIBITION
BKC ² 20hrs/37°C	1.500	93
BKC 20hrs/37°C	0.750	97
BKC 20hrs/37°C	0.375	92
BKC 4 hrs/37°C	1.500	86
BKC 4 hrs/37°C	0.750	89
BKC 4 hrs/37°C	0.375	87
BKC+Pronase™ 20hrs/37°C boiled	1.500	-19
BKC+Pronase™ 20hrs/37°C boiled	0.750	-6
BKC+Pronase™ 20hrs/37°C boiled	0.375	-7
BKC+Pronase™ 4 hrs/37°C boiled	1.500	-4

Table 1 (continued)

BKC+Pronase™ 4 hrs/37°C boiled	0.750	-7
BKC+Pronase™ 4 hrs/37°C boiled	0.375	-17...
Pronase™ 20hrs/37°C boiled self-digested	0.150	-29
Pronase™ 20hrs/37°C boiled self-digested	0.075	-21
Pronase™ 20hrs/37°C boiled self-digested	0.037	-22
Pronase™ 4 hrs/37°C boiled self-digested	0.150	-8
Pronase™ 4 hrs/37°C boiled self-digested	0.075	-34
Pronase™ 4 hrs/37°C boiled self-digested	0.037	-64

¹Comparison of inhibition by bovine κ -casein, hydrolyzed κ -casein, and protease (Pronase™) alone.

²Bovine kappa-casein

Table 2

INHIBITION OF HUMAN ROTAVIRUS INFECTION BY BOVINE KAPPA-CASEIN:
EFFECT OF HYDROLYSIS¹

TREATMENT	CONCENTRATION mg/ml	PERCENT INHIBITION
BKC+Pronase™ 20hrs/37°C boiled	1.5000	10
BKC+Pronase™ 20hrs/37°C boiled	0.7500	6
BKC+Pronase™ 20hrs/37°C boiled	0.3750	-6
Pronase™ 20hrs/37°C boiled self-digested	0.1500	54
Pronase™ 20hrs/37°C boiled self-digested	0.0750	13
Pronase™ 20hrs/37°C boiled self-digested	0.0375	8
Pronase™ 20hrs/37°C boiled self-digested	0.0188	8
Pronase™ 20hrs/37°C boiled self-digested	0.0094	15

¹Comparison of hydrolyzed bovine κ -casein and protease (Pronase™) alone.

EXAMPLE 11: Utility and Administration of Kappa-Casein

Human κ -casein can be extracted from human milk or can be obtained by recombinant DNA methods and cloning in prokaryotic or eukaryotic cells or from transgenic mammals using the DNA sequence and expression system of Hansson et al. Bovine κ -casein can be extracted from bovine milk where it is present at a concentration of approximately 3.3 grams/liter.

A DNA sequence encoding the human milk protein κ -casein is disclosed in PCT Publication No. WO 93/15196 (Hansson et al., "DNA Encoding Kappa- Casein, Process for Obtaining the Protein and Use Thereof") which is being incorporated herein by reference for the purpose of teaching a method of constructing an expression system for a DNA sequence encoding a polypeptide having an amino acid sequence which has the biological activity of human κ -casein. The DNA sequence is shown in Fig. 8 (SEQ ID NO: 1:) and the amino acid sequence of the polypeptide which it encodes is shown in Fig. 9 (SEQ ID NO: 2:). The encoded polypeptide was determined to have the antimicrobial activity or opioid activity of human κ -casein. The DNA sequence shown can be used in the production of recombinant human κ -casein using either a procaryotic or a eukaryotic system, or can be used to produce transgenic non-human mammals. Recombinant human κ -casein can be used as a constituent of infant formula to improve the nutritional and biological value of the formula or, as in the case of the present invention, to provide protection against enteric infection by human rotavirus.

The DNA sequence disclosed by Hansson et al. was determined from a cDNA clone isolated from a human mammary gland cDNA library. Construction of the expression system and its molecular biological characterization employed standard recombinant DNA methods. The procedure used was as follows: *E. coli* Y1090 bacteria were grown on LB (Luria-Bertoni) plates containing 50 μ g/ml of carbenicillin. A single colony was isolated and grown overnight in a LB containing 0.2% maltose and 10 mM $MgSO_4$. 0.4 ml of the culture was then mixed with diluted library phag s and

adsorption was allowed for 15 minutes at 37°C. The infected culture was mixed with 7 ml soft agarose (0.75% agarose in LB and 10 mM MgSO₄). The soft agarose mixture was poured on 150 mm LB plates. The plates were incubated at 42°C for about 3.5 hours until plaques were visible. Thereafter, each plate was overlaid with a membrane (DuPont NEN®, Colony Plaque Screen) previously saturated in 10 mM IPTG (Isopropyl-β-D-thiogalactoside), and incubated overnight at 37°C. The positions of the membranes were indicated before the membranes were removed. The membranes were washed in TTBS (tris buffered saline (TBS) containing 0.05% Tween 20), and incubated in TTBS containing 20% FCS and κ-casein antisera which had been raised in rabbits, purified, and diluted 1:25 for 2 hours at room temperature. The membranes were washed two times for 5 minutes in TTBS at room temperature. Biotinylated goat-antirabbit IgG in TBS (50mM Tris-HCL pH 7.9, 150 mM NaCl) was added and the membranes incubated for 1 hour at room temperature. The membranes were washed again with TTBS two times for 5 minutes at room temperature. The conjugate of streptavidin and biotinylated alkaline phosphatase in TTBS was added followed by an incubation for 1 hour at room temperature. The membranes were then washed four times in TTBS for 5 minutes and rinsed three times in a buffer containing 50 mM Tris-HCl pH 9.8, 3 mM MgCl₂, 50 μg/ml XP (5-bromo-4-chloro-3-indoylphosphate) and 100 μg/ml NBT (Nitroblue tetrasolium grade III). Approximately 100 positive plaques were identified.

The isolated plaques were purified by dilution and repeated screening. Phage DNA was prepared and the DNA preparations were digested with EcoRI. The digested DNA was separated by agarose electrophoresis and a number of EcoRI fragments were cloned into EcoRI digested and alkaline phosphatase treated pUC 18 plasmids and subsequently transformed into *E. coli* TG2. Transformants were selected on plates containing 50 μg/ml of carbenicillin, 40 μg/ml of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) and 1 mM IPTC (Isopropyl-β-D-thiogalactoside). Plasmid DNA was analyzed from a number of transformants. A transformant

harboring a plasmid containing the full-length cDNA fragment encoding human κ -casein was designated pS 270. Plasmid pS 70 DNA was subjected to restriction endonuclease analysis. The complete nucleotide sequence of both strands of the region encoding κ -casein was determined using a T7 sequencing kit (Pharmacia, Uppsala, Sweden) on double stranded templates as described by the vendor. Specific oligonucleotides complementary to pUC 18 or κ -casein sequences were used as primers for sequencing reactions.

The nucleotide sequence contained an open reading frame sufficient to encode the entire amino acid sequence of a κ -casein precursor protein consisting of 162 amino acids and a signal peptide of 20 amino acids.

Plasmid DNA, designated pS 270 was deposited in the collection of Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1 b, D.3300 Braunschweig, Germany and is identified by accession #DSM 6878.

Purified human or bovine κ -casein or their derivatives can be administered enterally in the form of an oral mucosal dosage unit or with a feeding tube, eg. nasojejunal or jejunal. In oral use, κ -casein should be between 2% and 50% by weight of the preparation. In pharmaceutical preparations, κ -casein should be mixed with a solid pulverulent carrier such as lactose, sorbitol, starch, or gelatin and pressed into tablet form. Multiple-unit dosages can also be prepared. Tablets and granules can be coated with substances such as polymers which change the dissolution rate in the gastrointestinal tract. Examples of coatings include anionic polymers having a pKa of above 5.5. Liquid preparations of human or bovine κ -casein can be prepared as 0.2% by weight of the active compound and glycerol. The daily dose of the active compound will vary with the administrative route.

The discovery disclosed herein of the antiviral activity of the secretory milk protein, κ -casein, makes feasible the use of the agent in infant formula, in pharmaceutical substances useful for the prevention and treatment of rotaviral infection in all groups at risk, as a nutritional supplement for patients

undergoing antibiotic therapy, and as an ingredient in other bioactive enteral nutritional products. This list is meant to be suggestive and should not be interpreted as limiting the potential uses of κ -casein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Abbott Laboratories
- (ii) TITLE OF INVENTION: Inhibition of Human Rotavirus Infection
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Donald O. Nickey
ROSS Products Division
Abbott Laboratories
 - (B) STREET: 625 Cleveland Avenue
 - (C) CITY: Columbus
 - (D) STATE: Ohio
 - (E) COUNTRY: United States of America
 - (F) ZIP: 43215
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb storage
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: MS-DOS Version 6.21
 - (D) SOFTWARE: WordPerfect Version 6.0a
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/308,882
 - (B) FILING DATE: 16-SEP-1994
 - (A) APPLICATION NUMBER: US 08/308,883
 - (B) FILING DATE: 16-SEP-1994
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (614) 624-3774
 - (B) TELEFAX: (614) 624-3074
 - (C) TELEX: None

(2) INFORMATION FOR SEQ ID NO: 1

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 857 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: cDNA
 - (A) DESCRIPTION: Human milk kappa-casein
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE:
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE: Human
 - (A) ORGANISM: Homo sapiens
 - (B) STRAIN:
 - (C) INDIVIDUAL ISOLATE:
 - (D) DEVELOPMENTAL STAGE: Adult
 - (E) HAPLOTYPE:
 - (F) TISSUE TYPE: Mammary gland
 - (G) CELL TYPE:
 - (H) CELL LINE:
 - (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE: Human Mammary Gland
 - (A) LIBRARY:
 - (B) CLONE:

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 45...593

(C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis

(D) OTHER INFORMATION: The encoded product of nucleotide SEQ ID NO: 1 is the human milk protein, kappa-casein, having the amino acid sequence shown in SEQ ID NO: 2.

(x) PUBUBLICATION INFORMATION:

(A) AUTHORS: L. Hansson, et al.

(B) TITLE: DNA Encoding Kappa-Casein, Process for Obtaining the Protein and Use Thereof

(C) JOURNAL:

(D) VOLUME:

(E) ISSUE:

(F) PAGES:

(G) DATE:

(H) DOCUMENT NUMBER: PCT/WO93/15196

(I) FILING DATE: 25-JAN-1993

(J) PUBLICATION DATE: 05-AUG-1993

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

GAATTCGAG AGAAGACCTG ACTGGCACGA GGAAAGGTGC AATA ATG AAG AGT
TTT 56

Met Lys Ser Phe

1

CTT CTA GTT GTC AAT GCC CTG GCA TTA ACC CTG CCT TTT TTG GCT GTG104

Leu Leu Val Val Asn Ala Leu Ala Leu Thr Leu Pro Phe Leu Ala Val
5 10 15 20

GAG GTT CAA AAC CAG AAA CAA CCA GCA TGC CAT GAG AAT GAT GAA AGA
152

Glu Val Gln Asn Gln Lys Gln Pro Ala Cys His Glu Asn Asp Glu Arg
25 30 35

CCA TTC TAT CAG AAA ACA GCT CCA TAT GTC CCA ATG TAT TAT GTG CCA
200

Pro Phe Tyr Gln Lys Thr Ala Pro Tyr Val Pro Met Tyr Tyr Val Pro
40 45 50

AAT AGC TAT CCT TAT TAT GGA ACC AAT TTG TAC CAA CGT AGA CCA GCT
248

Asn Ser Tyr Pro Tyr Tyr Gly Thr Asn Leu Tyr Gln Arg Arg Pro Ala
55 60 65

ATA GCA ATT AAT AAT CCA TAT GTG CCT CGC ACA TAT TAT GCA AAC CCA
296

Ile Ala Ile Asn Asn Pro Tyr Val Pro Arg Thr Tyr Tyr Ala Asn Pro
70 75 80

GCT GTA GTT AGG CCA CAT GCC CAA ATT CCT CAG CGG CAA TAC CTG CCA
344

Ala Val Val Arg Pro His Ala Gln Ile Pro Gln Arg Gln Tyr Leu Pro
85 90 95 100

AAT AGC CAC CCA CCC ACT GTG GTA CGT CGC CCA AAC CTG CAT CCA TCA
392

Asn Ser His Pro Pro Thr Val Val Arg Arg Pro Asn Leu His Pro Ser
105 110 115

TTT ATT GCC ATC CCC CCA AAG AAA ATT CAG GAT AAA ATA ATC ATC CCT
440

Phe Ile Ala Ile Pro Pro Lys Lys Ile Gln Asp Lys Ile Ile Ile Pro
120 125 130

ACC ATC AAT ACC ATT GCT ACT GTT GAA CCT ACA CCA GCT CCT GCC ACT

488
 Thr Ile Asn Thr Ile Ala Thr Val Glu Pro Thr Pro Ala Pro Ala Thr
 135 140 145
 GAA CCA ACG GTG GAC AGT GTA GTC ACT CCA GAA GCT TTT TCA GAG TCC
 536
 Glu Pro Thr Val Asp Ser Val Val Thr Pro Glu Ala Phe Ser Glu Ser
 150 155 160
 ATC ATC ACG AGC ACC CCT GAG ACA ACC ACA GTT GCA GTT ACT CCA CCT
 584
 Ile Ile Thr Ser Thr Pro Glu Thr Thr Thr Val Ala Val Thr Pro Pro
 165 170 175 180
 ACG GCA TAAAAACACC AAGGAAATAT CAAAGAACAC AACGCAGGAC
 TTGCTGAAAC 640
 Thr Ala
 CAAATTACTA CTTCACACTC TCCTTCAGCC ATTTGTCTGC CTTCAGTCAA
 CAGAAAATGT700
 GATTTTCACA GATTCAGCTC TTCTCTCCTT ACATTTTACA TTCATGCCAC
 ATCAATATT 760
 TTGATTCTTG CACAATAAAG CCAACTGATT GCAAAAAAAAA AAAAAAAAAA
 AAAAAAAAAA 820
 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA CGAATTC
 857

(2) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 182 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Protein

(A) DESCRIPTION:

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STAGE:

(E) HAPLOTYPE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(H) CELL LINE:

(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION:

(x) PUBUBLICATION INFORMATION:

(A) AUTHORS: L. Hansson, et al.

(B) TITLE: DNA Encoding Kappa-Casein, Process for Obtaining the Protein and Use Thereof

(C) JOURNAL:

(D) VOLUME:

(E) ISSUE:

(F) PAGES:

(G) DATE:

(H) DOCUMENT NUMBER: PCT/WO93/15196

(I) FILING DATE: 25-JAN-1993

(J) PUBLICATION DATE: 05-AUG-1993

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

Met Lys Ser Phe Leu Leu Val Val Asn Ala Leu Ala Leu Thr Leu Pro
1 5 10 15

Phe Leu Ala Val Glu Val Gln Asn Gln Lys Gln Pro Ala Cys His Gin
 20 25 30

Asn Asp Glu Arg Pro Phe Tyr Gln Lys Thr Ala Pro Tyr Val Pro Met
 35 40 45

Tyr Tyr Val Pro Asn Ser Tyr Pro Tyr Tyr Gly Thr Asn Leu Tyr Gln
50 55 60

Arg Arg Pro Ala Ile Ala Ile Asn Asn Pro Tyr Val Pro Arg Thr Tyr
65 70 75 80

Tyr Ala Asn Pro Ala Val Val Arg Pro His Ala Gln Ile Pro Gln Arg
85 90 95

Gln Tyr Leu Pro Asn Ser His Pro Pro Thr Val Val Arg Arg Pro Asn
100 105 110

Leu His Pro Ser Phe Ile Ala Ile Pro Pro Lys Lys Ile Gln Asp Lys
115 120 125

Ile Ile Ile Pro Thr Ile Asn Thr Ile Ala Thr Val Glu Pro Thr Pro
130 135 140

Ala Pro Ala Thr Glu Pro Thr Val Asp Ser Val Val Thr Pro Glu Ala
145 150 155 160

Phe Ser Glu Ser Ile Ile Thr Ser Thr Pro Glu Thr Thr Thr Val Ala
165 170 175

Val Thr Pro Pro Thr Ala
180

What is claimed is:

1. An enteral nutritional product comprising an anti-rotaviral infection effective amount of an agent selected from the group consisting of native or recombinant unhydrolyzed human kappa-casein and native or recombinant unhydrolyzed bovine kappa-casein at a concentration exceeding that found in human or bovine milk.

2. The product of claim 1, wherein the enteral nutritional product is infant formula.

3. A pharmaceutical composition comprising a therapeutically effective amount of an agent selected from the group consisting of native or recombinant unhydrolyzed human kappa-casein and native or recombinant unhydrolyzed bovine kappa-casein for the therapeutic or prophylactic treatment of infections of the gastrointestinal tract caused by human rotavirus.

4. A method of preventing or retarding the onset of or treating an infection of a mammalian cell caused by human rotavirus comprising contacting the cell with an effective amount of an agent selected from the group consisting of native or recombinant unhydrolyzed human kappa-casein and native or recombinant unhydrolyzed bovine kappa-casein.

5. A method of preventing or retarding the onset of or treating infantile gastroenteritis comprising treating an infant or child at risk or afflicted with the disease according to the method of claim 4.

6. The method of claim 4, wherein the subject is an immunodeficient patient.

FIG-1

PREPARATION OF RADIOLABELED HUMAN ROTAVIRUS (HRV)

MEDIUM CONTAINING ^{125}I AND CELL LYSATES FROM
HUMAN ROTAVIRUS INFECTED MA-104 CELLS ARE
COLLECTED AFTER 1 OR 2 DAYS INFECTION



EXTRACTION WITH TRICHLOROTRIFLUOROETHANE



CENTRIFUGATION AT 7000g FOR 30 MINUTES



ULTRACENTRIFUGATION ON 20%
SUCROSE CUSHION AT 90,000g FOR 3 HOURS



ULTRACENTRIFUGATION ON 20% TO 60%
SUCROSE GRADIENT AT 100,000g FOR 2.5 HOURS



FRACTIONS ARE COLLECTED AND VIRUS CONTAINING
FRACTIONS ARE IDENTIFIED BY COMPARING TO
RADIOLABELED VIRUS STANDARD OR
DETERMINED BY ELISA

FIG-2
INHIBITION OF BINDING OF ^{125}I -HRV WA
TO MA-104 CELLS

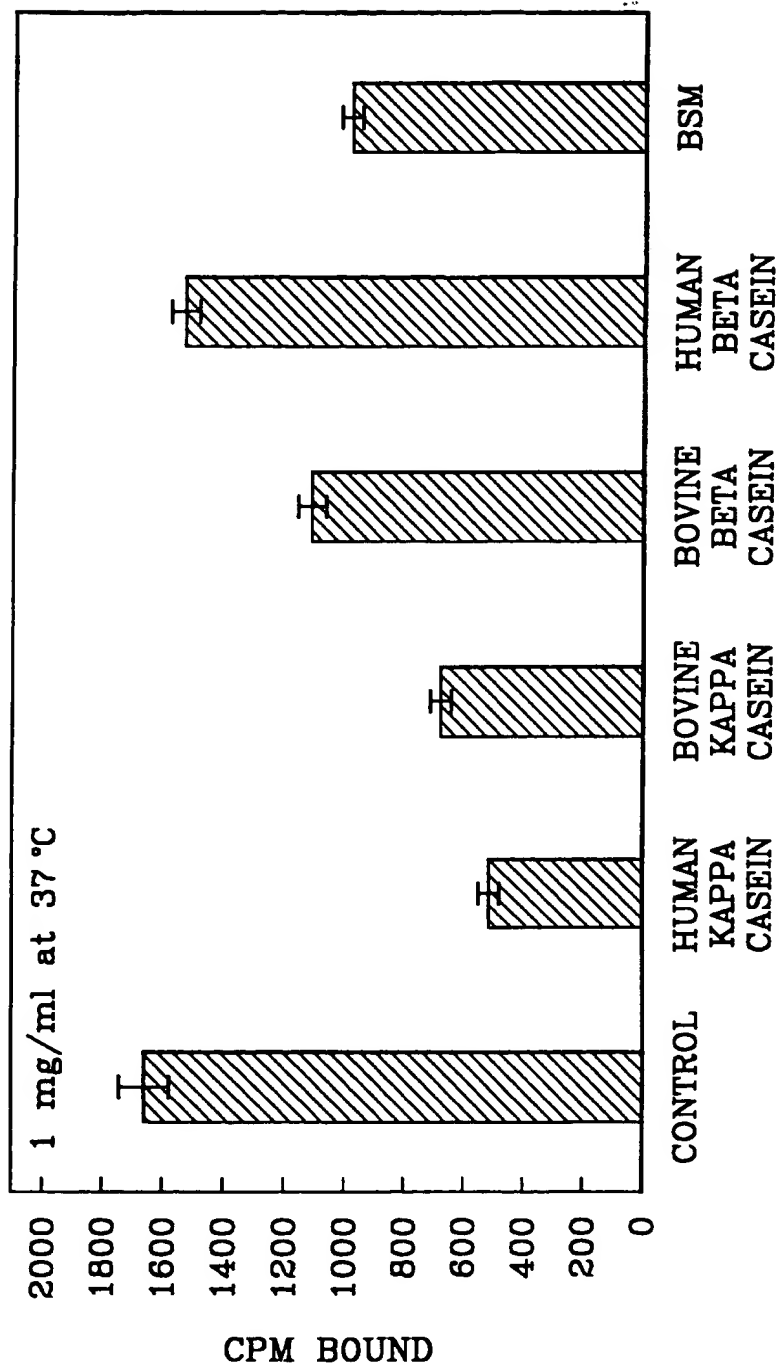
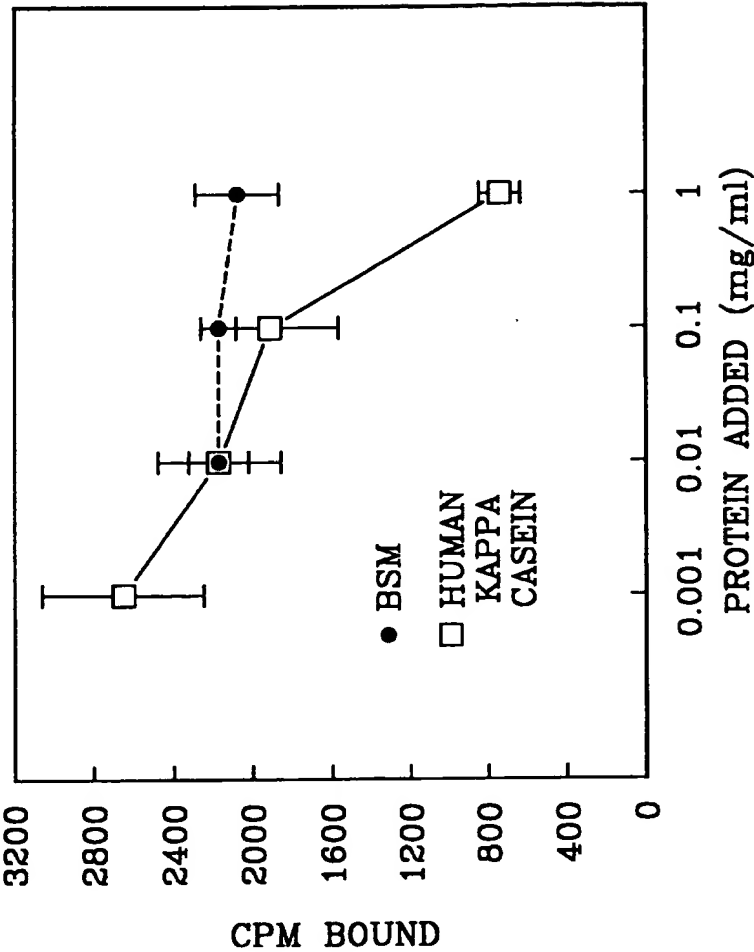


FIG-3
DOSE-DEPENDENT INHIBITION OF BINDING OF ^{125}I -HRV WA TO MA-104 CELLS



BSM: BOVINE SUBMAXILLARY MUCIN

FIG-4
INHIBITION OF BINDING OF ¹²⁵I-HRV WA
TO MA-104 CELLS BY EXOGLYCOSIDASE
TREATED HUMAN KAPPA CASEIN

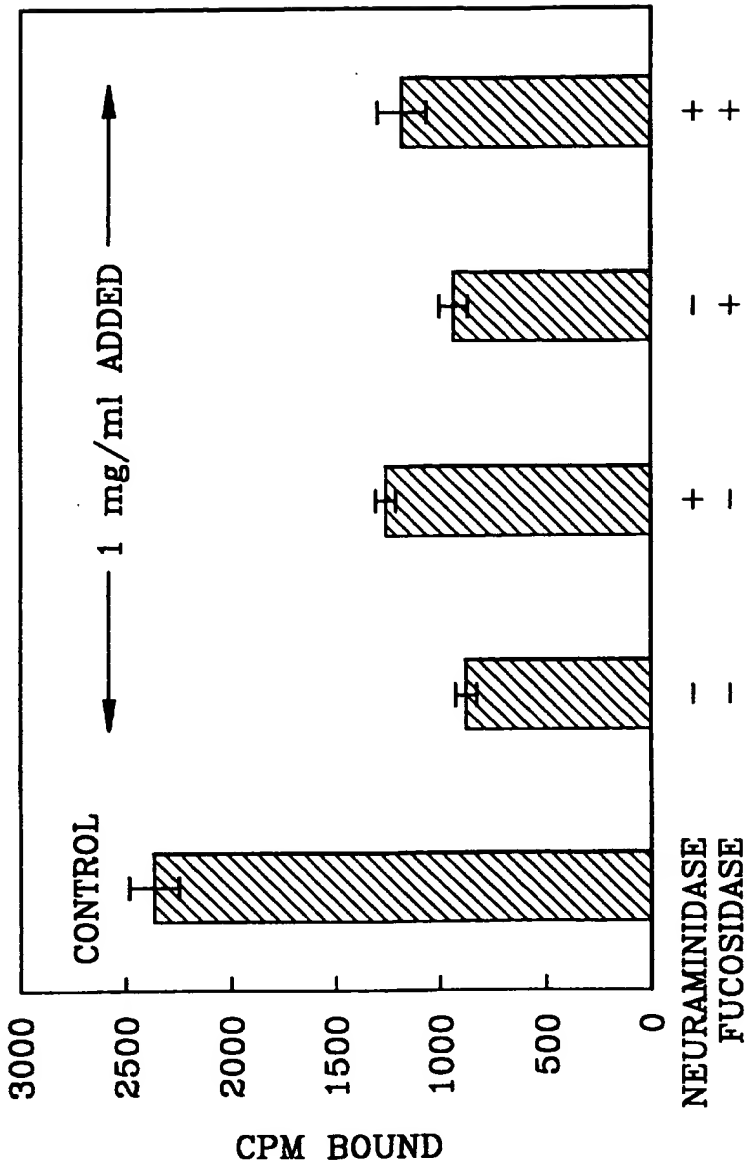


FIG-5
INHIBITION OF BINDING OF ^{125}I -HRV WA
TO MA-104 CELLS BY GLYCOPROTEINS
(100 nmole HEXOSES WERE ADDED TO EACH WELL)

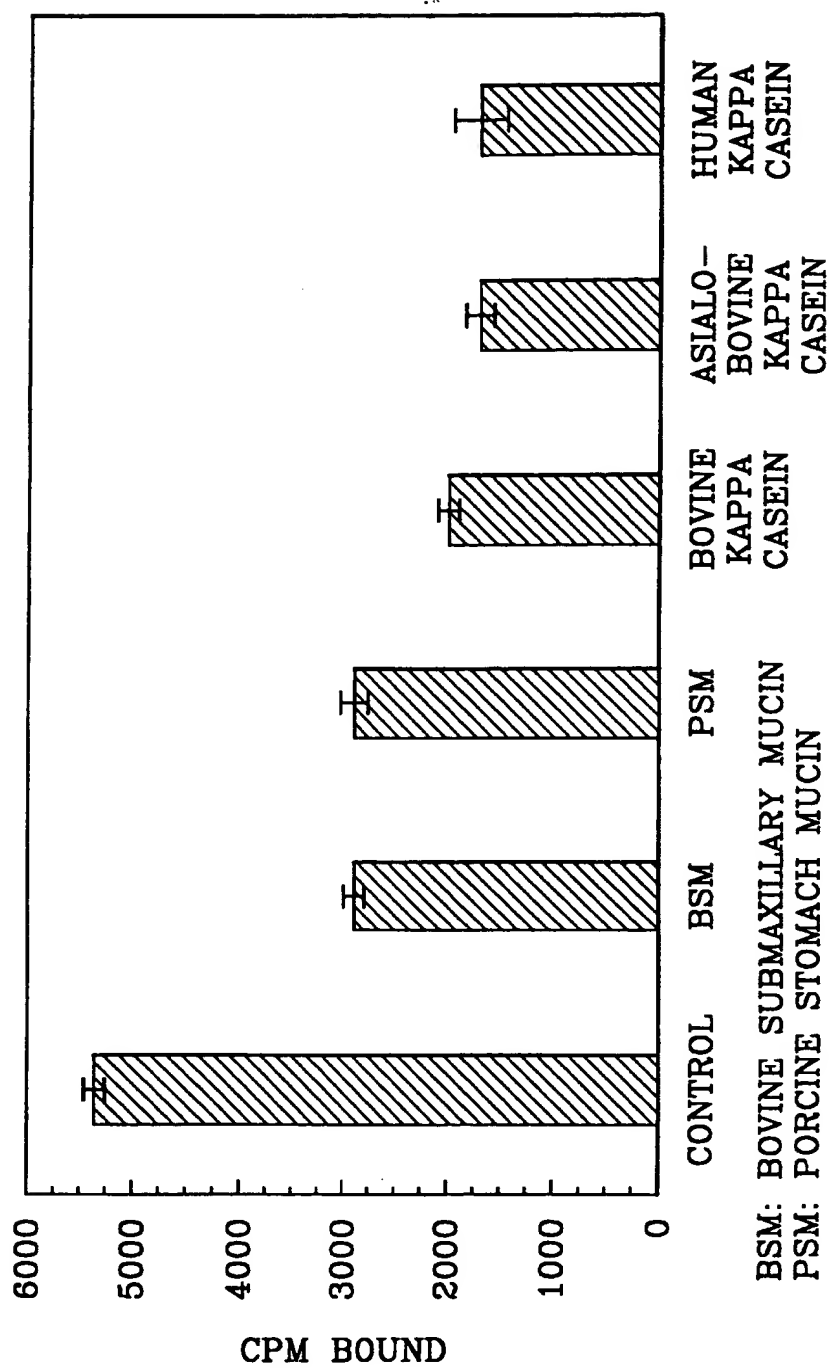


FIG-6
INHIBITION OF BINDING OF ^{125}I -HRV WA TO MA-104
CELLS BY NATIVE AND PRONASE® DIGESTED
BOVINE KAPPA CASEIN (BKC)

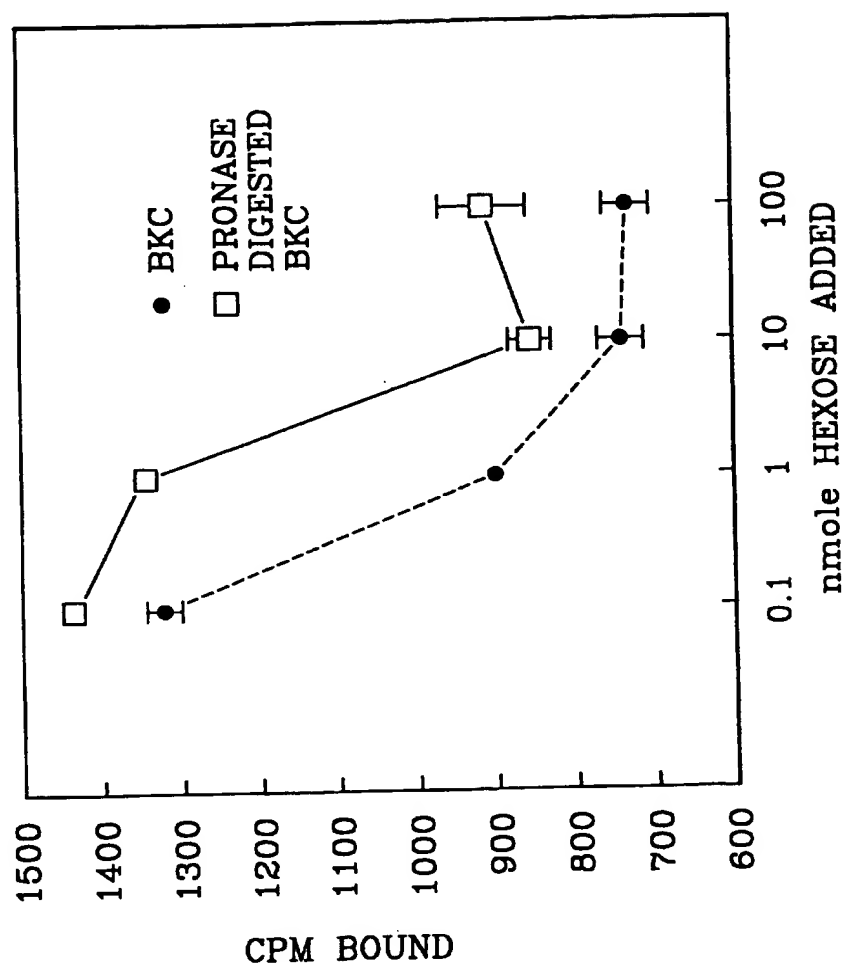


FIG-7
NEUTRALIZATION OF HUMAN ROTAVIRUS
BY BOVINE KAPPA CASEIN (BKC)

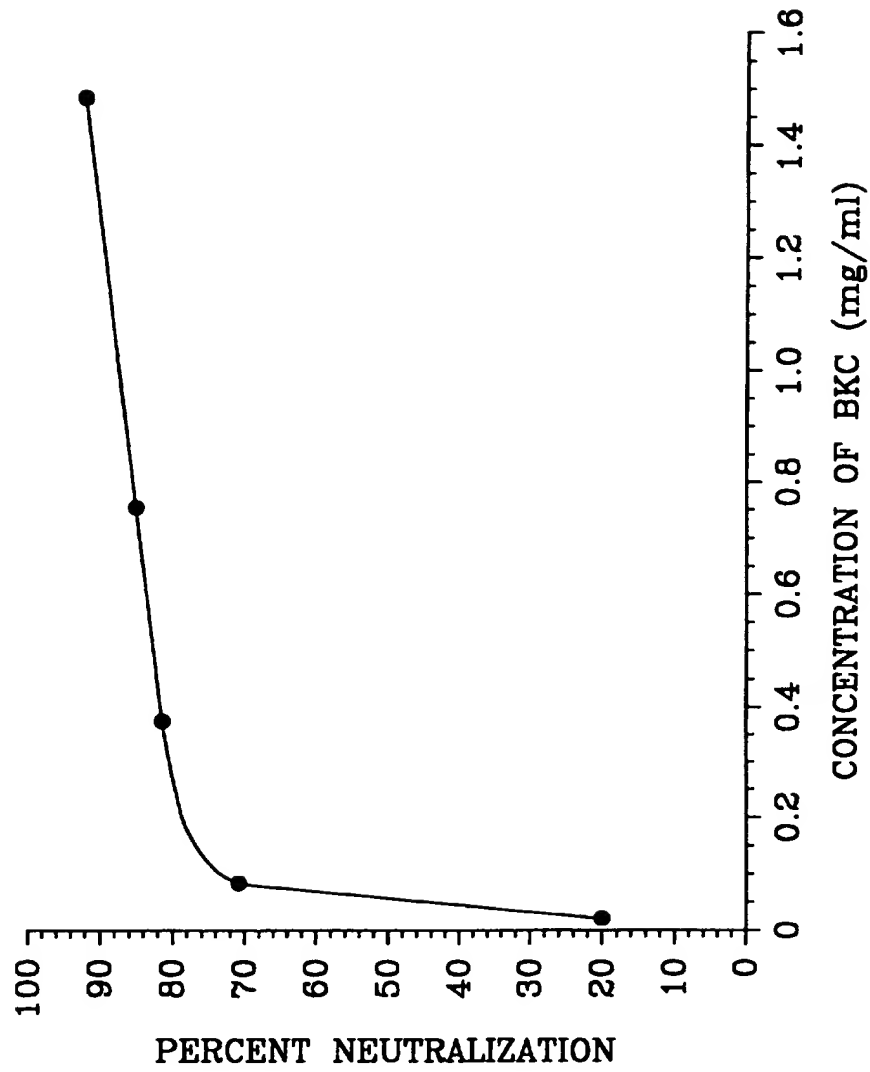


FIG-8

GAATTCGAG AGAAGACCTG ACTGGCACGA GGAAAGGTGC AATA ATG AAG AGT TTT 56
Met Lys Ser Phe
1

CTT CTA GTT GTC AAT GCC CTG GCA TTA ACC CTG CCT TTT TTG GCT GTG 104
Leu Leu Val Val Asn Ala Leu Ala Leu Thr Leu Pro Phe Leu Ala Val
5 10 15 20

GAG GTT CAA AAC CAG AAA CAA CCA GCA TGC CAT GAG AAT GAT GAA AGA 152
Glu Val Gln Asn Gln Lys Gln Pro Ala Cys His Glu Asn Asp Glu Arg
25 30 35

CCA TTC TAT CAG AAA ACA GCT CCA TAT GTC CCA ATG TAT TAT GTG CCA 200
Pro Phe Tyr Gln Lys Thr Ala Pro Tyr Val Pro Met Tyr Tyr Val Pro
40 45 50

AAT AGC TAT CCT TAT TAT GGA ACC AAT TTG TAC CAA CGT AGA CCA GCT 248
Asn Ser Tyr Pro Tyr Tyr Gly Thr Asn Leu Tyr Gln Arg Arg Pro Ala
55 60 65

ATA GCA ATT AAT AAT CCA TAT GTG CCT CGC ACA TAT TAT GCA AAC CCA 296
Ile Ala Ile Asn Asn Pro Tyr Val Pro Arg Thr Tyr Tyr Ala Asn Pro
70 75 80

GCT GTA GTT AGG CCA CAT GCC CAA ATT CCT CAG CGG CAA TAC CTG CCA 344
Ala Val Val Arg Pro His Ala Gln Ile Pro Gln Arg Gln Tyr Leu Pro
85 90 95 100

AAT AGC CAC CCA CCC ACT GTG GTA CGT CGC CCA AAC CTG CAT CCA TCA 392
Asn Ser His Pro Pro Thr Val Val Arg Pro Asn Leu His Pro Ser
105 110 115

TTT ATT GCC ATC CCC CCA AAG AAA ATT CAG GAT AAA ATA ATC ATC CCT 440
Phe Ile Ala Ile Pro Pro Lys Lys Ile Gln Asp Lys Ile Ile Ile Pro
120 125 130

ACC ATC AAT ACC ATT GCT ACT GTT GAA CCT ACA CCA GCT CCT GCC ACT 488
Thr Ile Asn Thr Ile Ala Thr Val Glu Pro Thr Pro Ala Pro Ala Thr
135 140 145

GAA CCA ACG GTG GAC AGT GTA GTC ACT CCA GAA GCT TTT TCA GAG TCC 536
Glu Pro Thr Val Asp Ser Val Val Thr Pro Glu Ala Phe Ser Glu Ser
150 155 160

ATC ATC ACG AGC ACC CCT GAG ACA ACC ACA GTT GCA GTT ACT CCA CCT 584
Ile Ile Thr Ser Thr Pro Glu Thr Thr Thr Val Ala Val Thr Pro Pro
165 170 175 180

ACG GCA TAAAAACACC AAGGAAATAT CAAAGAACAC AACGCAGGAC TTGCTGAAAC 640
Thr Ala

CAAATTACTA CTTCACTC TCCTTCAGCC ATTTGTCTGC CTTCACTCAA CAGAAAATGT 700

GATTTTCACA GATTCAGCTC TTCTCTCCTT ACATTTTACA TTCATGCCAC ATTCAATATT 760

TTGATTCTTG CACAATAAAG CCAACTGATT GCAAAAAAAA AAAAAAAAAA AAAAAAAAAA 820

AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA CGAATTC 857

[illegible]

INTERNATIONAL SEARCH REPORT

 Int. Appl. No.
 PCT/US 95/05676

 A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 A61K38/17 A23L1/305 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

 Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 A61K A23L C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 291 265 (SNOW BRAND MILK PRODUCTS) 17 November 1988 see the whole document ---	1-6
X	EP,A,0 488 589 (SNOW BRAND MILK PRODUCTS) 3 June 1992 see the whole document ---	1-6
X	WO,A,93 15196 (SYMBICOM AKTIEBOLAG) 5 August 1993 see claims 42,43,51-53 ---	1-6
X	WO,A,94 09651 (CANCER RESEARCH FUND OF CONTRA COSTA) 11 May 1994 see the whole document ---	1-6
-/--		

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *A* document member of the same patent family

Date of the actual completion of the international search

24 July 1995

Date of mailing of the international search report

22.08.95

Name and mailing address of the ISA

 European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Theuns, H

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 95/05676

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO, A, 94 15952 (NOVO NORDISK A/S) 21 July 1994 see the whole document -----</p>	1-6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/05676

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 4-6 are directed to a method of treatment of the human/animal body the search has been based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Int. No. and Publication No
PCT/US 95/05676

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0291265	17-11-88	JP-A- 63284133 US-A- 5147853 US-A- 5344820	21-11-88 15-09-92 06-09-94
EP-A-0488589	03-06-92	JP-A- 4198198 AU-B- 639620 AU-A- 8812391 DE-D- 69102401 DE-T- 69102401 US-A- 5278288	17-07-92 29-07-93 11-06-92 14-07-94 27-10-94 11-01-94
WO-A-9315196	05-08-93	CA-A- 2128110 EP-A- 0625197 JP-T- 7503136	05-08-93 23-11-94 06-04-95
WO-A-9409651	11-05-94	AU-B- 5896194	24-05-94
WO-A-9415952	21-07-94	AU-B- 5832994	15-08-94